Use of RNAi in *Brachionus manjavacas* to Inhibit Cold-Related Genes Implicated in Aging

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Use of RNAi in *Brachionus manjavacas* to Inhibit Cold-Related Genes Implicated in Aging

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SUMMARY

Aging can be affected by a number of factors, including temperature; for example, organisms tend to live significantly longer when exposed to colder temperatures. Previous studies suggest that this change in life expectancy due to temperature change has a genetic component. Through the use of RNA interference, we have found that aging genes can be knocked-down in our model animal, *Brachionus manjavacas* (Rotifera). Using RNAi, we examined the effect of genetic knock-down on genes related to life extension at lower temperatures (16^{o}C) compared to standard culture temperatures (22^{o}C). This study has provided evidence that temperature-dependent changes in longevity may be largely due to changes in expression levels in select genes: Forkhead Box C (FhBC), TRP7, and S6P. Future research may show that the life extending effects of certain living conditions may be obtainable through genetic treatment.

CHAPTER 1

INTRODUCTION

The mechanism and rate of aging have been ongoing topics of interest in the scientific community due to their relevance for animal health, in particular, humans. Aging rate has been shown to be altered by a number of acute stressors such as heat stress, caloric restriction, and UV exposure (Haigis and Yankner 2010). Caloric restriction, in particular, is a widely studied stressor that can extend life in nearly all members of Animalia tested (Masoro 2006) though there are cases in which mutants, such as in inbred mice, can actually exhibit lower lifespan suggesting that there is a genetic component to these effects (Liao et al. 2010). There have been a number of proposed causes of aging including oxygen radical production in mitochondria (Barja 2004). The effects of reactive oxygen species (ROS) on aging are well accepted and further supported by the effect of caloric restriction which seems to extend life while decreasing the amount of free radicals present in the treated animal (Barja 2004). This alone, however, cannot explain why mutations would lead to a higher or lower lifespan.

Several questions remain as to whether all of these aging effects can be attributed solely to ROS or other simple chemical principles within the organism (Lee and Kenyon 2009, Xiao et al. 2013). For example, caloric restriction is thought to also be linked to lower internal body temperatures as was demonstrated in mice (Conti et al. 2006). Ali et al. investigated the effect of temperature change on the production of free radicals, but found that while a decrease in temperature increased lifespan, it also increased the release of free radicals, refuting the idea that ROS alone could be responsible for aging (Ali et al.2010). The presence of other aging mechanisms clearly needs further investigation.

In addition to acute stressors such as heat shock, long term changes in temperature have been shown to have a strong effect on life expectancy (Xiao et al. 2013). The prospect of lower temperature being linked to life extension in caloric restriction experiments was investigated because caloric restriction led to lower internal temperatures in these organisms (Conti et al. 2006). This hypothesis warrants further investigation because temperature may be an even more powerful regulator of lifespan.

Several authors argue that the change in life expectancy cannot be explained solely by thermodynamic processes that moderate metabolism with change in temperature (Xiao et al. 2013). Lee and Kenyon investigated how aging is related to biological factors and found that decreased life span in response to higher temperatures may be partially due to thermosensory neurons (2009). Similarly, Xiao et al. further investigated genes related to life extension due to temperature, but focused on the genetic mechanisms involved in life extension at low temperatures (2013). The authors found that manipulation through genetics alone could produce a change in life expectancy (Xiao et al. 2013). Gene expression in this experiment was altered by mutations found to be critical in the regulation of life span in relation to temperature (Xiao et al. 2013). This study provided insight into the impact of thermosensitive pathways like the TRP channel, leading us to select TRP7 as a target for RNAi.

Organismal response to stressors can lead to changes in gene expression to cope with new conditions (Spriggs et al. 2010). Because of this, it is thought that genetic manipulation can mimic the effects of these stressors under normal conditions. The investigation of gene function and their effects on aging can be examined through the use of gene knock-down technology. Double-stranded RNA (dsRNA) can be used silence or

inhibit gene expression in a protocol known as RNA interference (RNAi); in response to dsRNA, dicers can inhibit the translation of messenger RNA (mRNA) into proteins thus preventing the expression of a selected gene (Paddison et al. 2002). Utilizing this response, we can further investigate the effect of genetic pathways in response to lower temperatures. By investigating these genes individually, it may be possible to mimic the effects on life expectancy without changing stressor conditions.

Additionally, new data from our lab shows similar survival curves between animals treated at a 16°C and those transferred from 16°C to 22°C after the first 4 days of their lives (figure 1). The persistence of this effect beyond the treatment time provides new insight regarding the regulation of genetic pathways in response to changes in temperature and a variety of new conditions to test cold-induced aging genes.

CHAPTER 2

METHODS

The animals used in this experiment were hatched from *Brachionus manjavacas* eggs cultivated in the lab. The eggs were hatched in approximately 25ml 15ppt artificial salt water made from instant ocean under constant light at 25°C. Once hatched, animals for life tables were fed a mobile algae species, *Tetraselmis suecica*, and treated with 5-fluoro-2-deoxyuridine (FDU) to prevent the hatching of asexually produced eggs (Snell et al. 2012).

We first selected genes that were potentially related to the regulatory changes undergone by animals exposed to different temperatures. Transient Receptor Potential Cation Subfamily Member 7 (TRP7) was selected from a paper (Xiao et al. 2013) which identified cold-related genes in the TRP pathway using mutant *C. elegans*. Additionally, Forkhead box C-like protein (FhBC) was selected due to its similarity to genes in the TRPA pathway as identified by Xiao et al. (2013) and has shown life-extending properties in other experiments (Wang et al. 2001). Ribosomal Protein S6 Polypeptide 2 (S6P) was also selected due to its similarity to gens within the TRPA pathway and has been linked to change in life expectancy due to caloric restriction and in glycogen and insulin regulation (Eldar-Finkelman et al.1995; Cross et al. 1995). These genes and their associated primers are discussed in more detail in table 1.

We then explored how these genes can affect aging rates in a variety of experimental conditions. The selected genes were isolated using primers specific to those regions and amplified through PCR and gel electrophoresis (20 minutes at 120V in 2%

agarose). After this verification, T7 versions of these primers were used to produce dsRNA for the genes using MEGAscript's RNAi Kit. The DNA fragments produced by these primers contain T7 tags which allowed us to create dsRNA for gene knock-down via RNAi. Gel electrophoresis was then used to help verify whether bands of the appropriate size for our target dsRNA were present in our samples (figure 5). Newly hatched animals were transfected for 4 hours with dsRNA treated with FuGene6 transfection reagent. By doing this, we attempted to knock-down the genes of interest through the use of RNAi under three conditions (16^{o}C, 22^{o}C, and 4-days at 16^{o}C before transfer to 22^{o}C) to get a better idea of how these genes affect life expectancy under a variety of temperatures. To control for the transfection process, a genetic code not found in rotifers, TOR scramble, was used to create dsRNA that should not interfere with the regulation of genes within the transfected animals. The following RNAi screens are 15-day periods where treated animals and untreated animals are monitored for changes in life expectancy. Mortality is recorded each day to generate a survivorship curve. A successful treatment should negate the effects of 4-day cold treatment on animals so that their longevity resembles that of the animals treated at 22^{o}C rather than like animals treated at 16 ^{o}C as seen in the control (figure 1). This will show whether or not this gene contributes to the increase in life expectancy of an animal even after it has been removed from cold treatment.

qPCR verification should show that the results of the experiment were indeed because of the lowered expression of our selected gene. So far, only TRP7 has been tested using qPCR. Newly hatched animals were transfected with dsRNA of the targeted gene. After the transfections animals were collect at different times depending of the

treatment (0 hours, 24 hours, or 48 hours after transfection). Afterward, 10 animals per treatment were collected and suspended in RNA later with at least 8 tubes of animals. These tubes were kept at -80°C until use for qPCR. RNA was extracted from treated animals using the RNeasy Micro Kit and the RNeasy MinElute Cleanup Kit. qPCR was performed using the Express One-Step SYBR GreenER Kit. Each qPCR plate contained purified RNA from these animals (8 sets of TOR Scramble, 8 sets of the treatment gene each with three replicates). Actin acted as our housekeeping gene, a gene that is unchanged by RNAi treatments. The values generated through this analysis using the ΔΔCt method are presented as fold increases or decreases in the treatment group when compared to the control.

CHAPTER 3

RESULTS

Figure 1.

Figure 1 represents the control data used in this experiment for comparison to our test results. On the left side of the figure are values which denote a ratio of survival in the test subjects. At the bottom of the figure are numbers representing the number of days that the data was collected during the experiment. The colored lines represent the three different testing conditions. The green line represents animals that were kept at 16ºC. The red line represents animals that were kept at 22ºC which is the standard temperature that laboratories typically raise the animals. The blue line denotes animals that were kept at 16ºC for first four days of their lives and then transferred to an incubator kept at 22ºC for the remainder of their lives. As can be seen, animals raised at 16ºC live significantly longer than animals raised or kept at 22ºC as shown by the green line in Figure 1.

As seen in Figure 1, animals kept at 16ºC and then moved on day four of their life to an incubator kept at 22ºC, exhibited extended effects of the 16ºC control temperature until approximately day fifteen. At this point the animals show a marked decrease in longevity. After this decline, these animals' longevity matched the trajectory of animals kept at 22°C suggesting some return to normalcy after early 16°C treatment.

Figure 2.

Figure 2 shows the longevity of animals treated with TRP7 double stranded RNA. As can be seen, animals kept at 16ºC exhibit higher longevity than animals kept at 22ºC (depicted by the blue and green lines of the graph.) However, in this case, the animals

kept at 16ºC for the first four days of their life, and then moved to an incubator kept at 22ºC (denoted by the red line) show a much closer survivorship to the animals kept at 22ºC (blue line). This figure shows a significant difference between the control group of untreated rotifers and those treated with TRP7 double stranded RNA (22°C vs 16°C: $p = 0.0473$, 22°C vs 4d 16°C: $p= 0.0.3459$, 4d 16°C vs 16°C: $p = 0.0004$).

Figure 3.

Figure 3 shows the longevity of animals treated with FhBC double stranded RNA. As can be seen, animals kept at 16ºC exhibit higher longevity than animals kept at 22ºC (depicted by the blue and green lines of the graph.) Again, the animals depicted by the red line were kept at 16ºC for the first four days of their life, and then moved to an incubator kept at 22ºC. These rotifers (red line) demonstrated similar survivorship to the animals kept at 22ºC (blue line) (22°C vs 16°C: $p \leq 0.0001$, 22°C vs 4d 16°C: $p= 0.0.1280$, 4d 16°C vs 16°C: $p = 0.0019$). However, this decrease in the survivorship of the rotifers was slightly less pronounced than the result found in Figure 2.

Figure 4.

Figure 4 shows the longevity of animals treated with S6P double stranded RNA. As can be seen, animals kept at 16ºC exhibit higher longevity than animals kept at 22ºC (depicted by the blue and green lines of the graph.) The animals depicted by the red line, were again kept at 16ºC for the first four days of their life, and then moved to an incubator kept at 22ºC. These rotifers show similar survivorship to the animals kept at 22ºC (22°C vs 16°C: $p = 0.0473$, 22°C vs 4d 16°C: $p= 0.9948$, 4d 16°C vs 16°C: $p = 0.0401$). However, the difference between the animal subjects was much less pronounced than in the results found in Figure 3.

Figure 5.

PCR and gel verification showed that our primers were able to bind and produce dsRNA of appropriate sizes (~500 bp) for TOR Scramble, FhBC, S6P. and TRP7. Each set of primers was shown to produce single bands aside from what we suspect to be excess primer at lower band sizes (≤ 100bp).

Figure 6.

Animals were collected 0 hours, 24 hours, and 48 hours after transfection with dsRNA. qPCR analysis showed a dramatic fold increase in TRP7 expression in animals collected at 0 hours. The fold increase was insignificant in animals collected 24 hours and 48 hours after transfection.

Figure 7.

Because of the results of the qPCR data seen in figure 6, a second experiment was run on three different treatments of animals. Animals were raised in only artificial salt water (ASW), with food (*Tetraselmis suecica*), or with both *Tetraselmis suecica* and 5-fluoro-2-deoxyuridine (FDU). Animals raised in ASW exhibited a 50% increase in TRP7 expression after dsRNA treatment. Other treatments did not show significant change in TRP7 expression.

CHAPTER 4

DISCUSSION

In a study performed by Xiao, et al. (2013), it was found that an increase of longevity at lower temperatures is dependent on a thermosensitive TRP channel. Our study shown in Figure 2 supports Xiao's finding that a loss of longevity occurs when TRP7, a component of this TRP channel, is knocked-down in the subjects. These findings demonstrate that genetic factors may contribute to life extension at cold temperatures.

Forkhead Box genes (FOX) have been shown to reduce age-related proliferation defects in cells (Wang et al. 2001) as well as to affect oxidative processes related to age-related diseases (Manolagas & Almeida 2007). Forkhead Box genes and genes related to caloric restriction (i.e. Sirtuin family genes) have been shown to affect life expectancy, and have been scientifically implicated in aging processes (Birkenkamp & Coffer 2003).

The findings presented in figure 3 of our experiment show that longevity is decreased in animals where Forkhead Box C-like Protein has been knocked-down. This finding reinforces previous studies in that it demonstrates that Forkhead Box genes may be related to both caloric restriction and life extension in general. The findings in Figure 3 reinforce the hypothesis that caloric restriction, which has been shown in previous studies to extend life, may be related to a decrease in internal body temperature (Conti, et al. 2006).

Because of the connection between caloric restriction and internal body temperature (Conti, et al. 2006), glycogen and insulin regulating pathways and related genes, like Ribosomal Protein S6 Polypeptide 2 (S6P) (Eldar-Finkelman 1995), may

cause changes in longevity in relation to temperature. Figure 4 demonstrates that changes in the expression of S6P can cause changes in longevity in relation to temperature.

Because there was a decrease in the life extending effect in dsRNA-treated animals exposed to 16^{o}C for 4 days and then moved to the incubator kept at 22^{o} C, when compared to the control, it is likely that the genes of interest (TRP7, FhBC and S6P) were knocked down or altered in another way, thus negating the effect of increased longevity when exposed to lower temperatures early in life.

While we have yet to verify this knock-down with qPCR, we are hopeful that the current results suggest that the gene knock-down was successful. So far, attempted qPCR verification for TRP7 knock-down has not shown a significant decrease in expression level when compared to the control; in fact, animals collected 0 hours after transfection exhibited large increases in TRP7 (figure 6). This dramatic increase, while unexpected, could indicate the successful transfection of dsRNA that has yet to be digested by dicer enzymes. Because we have seen a decrease in life expectancy in animals treated with dsRNA for our selected genes, we believe that the mechanisms by which the transfections are affecting longevity may not be measurable by our current qPCR protocol. Exploration on the potential for 5-fluoro-2-deoxyuridine interference within the qPCR did not lead to enlightening results. Animals not treated with FDU still did not exhibit the expected knock-down of TRP7 expression (figure 7). qPCR analysis has not yet been done using animals transfected with FhBC or S6P dsRNA.

We intend to continue collecting life table data for a variety of genes and performing qPCR analysis for each treatment. Genes of interest will likely include both directly temperature-related genes and genes linked to decrease or increase in body

temperature due to caloric restriction or digestion. Previous research has shown that decreased internal body temperature in mice has also led to increased longevity (Conti et al. 2006) We hope that by identifying genes that affect longevity that these genes could potentially be targets for treatments in other target organisms to increase lifespan without a dramatic change in lifestyle or living conditions.

In addition to potential treatments using RNAi, it is possible that by exploring these pathways we can find new potential targets for the introduction of drugs to increase longevity in treated individuals. For instance, Drosophila animals treated with PBA have been shown to have significant increases in life expectancy without any other major changes in behavior by inducing genes of various natures including other neurotransmitters and genes related to metabolism (Kang et al. 2002). The application of these findings is in its infancy at this point in time. However, the implications of these studies are promising and may eventually lead to targeting genetic pathways to treat a variety of diseases.

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TABLES AND FIGURES

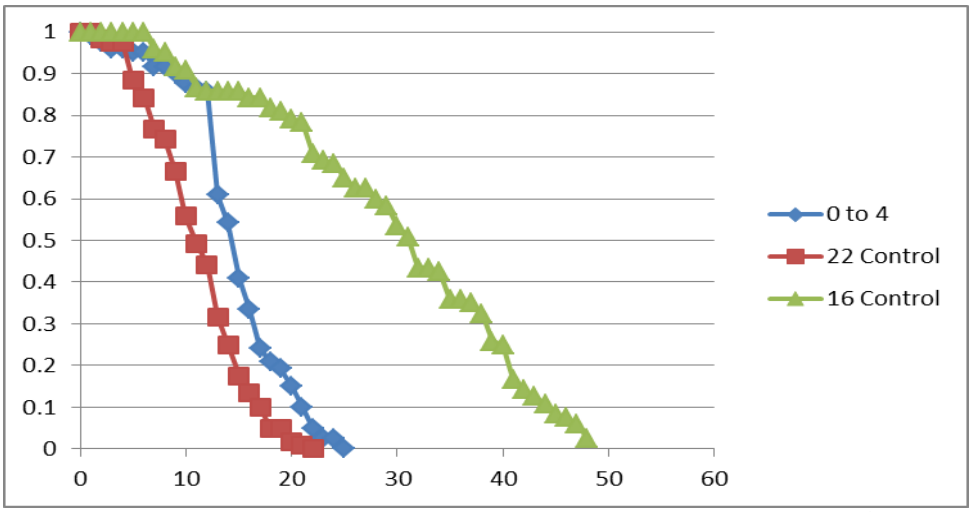

Figure 1: Three treatments are shown: animals exposed continuously to 16°C, animals exposed continuously to 22°C, and animals exposed to 16°C for 4 days then exposed to 22°C for the remainder. Animals exposed to 16°C from 0 to 4 days have a similar survivorship trajectory until age 12 days as that of animals cultured at 16°C for their entire lives. Both 16°C treatments have better survival than rotifers cultured at 22°C continuously.

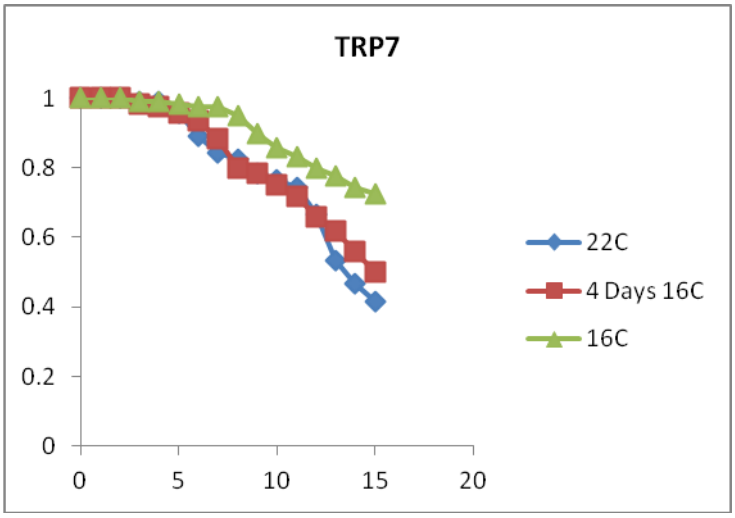

Figure 2. **(22C vs 16C: p = 0.0473, 22C vs 4d 16C: p= 0.0.3459, 4d 16C vs 16C: p = 0.0004)** This graph shows the longevity (in terms of days) of animals treated with double-stranded TRP7 RNA. Three treatments are shown: animals exposed continuously to 16°C, animals exposed continuously to 22°C, and animals exposed to 16°C for 4 days then exposed to 22°C for the remainder. The animals kept at 16ºC for the first four days of their life, and then moved to an incubator kept at 22ºC (denoted by the red line) show a closer survivorship to the animals kept at 22ºC (blue line) unlike the untreated animals in figure 1.

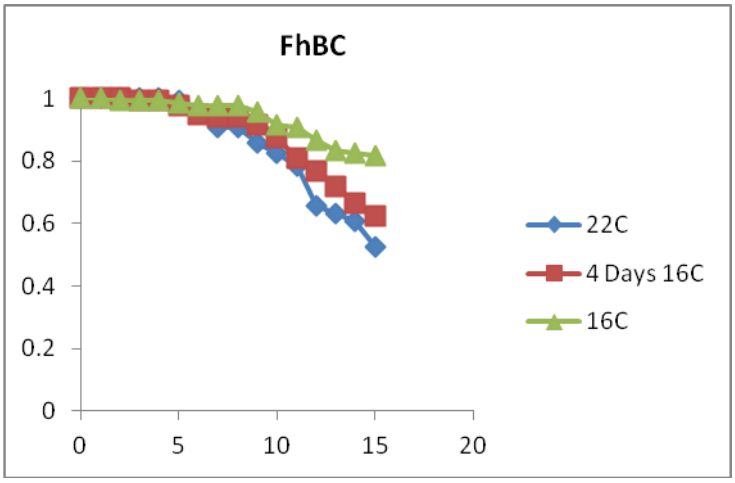

Figure 3. **(22C vs 16C: p ≤ 0.0001, 22C vs 4d 16C: p= 0.0.1280, 4d 16C vs 16C: p = 0.0019)** This graph shows the longevity (in terms of days) of animals treated with double-stranded FhBC RNA. Three treatments are shown: animals exposed continuously to 16°C, animals exposed continuously to 22°C, and animals exposed to 16°C for 4 days then exposed to 22°C for the remainder. The animals kept at 16°C for the first four days of their life, and then moved to an incubator kept at 22°C (denoted by the red line) show a closer survivorship to the animals kept at 22°C (blue line) unlike the untreated animals in figure 1.

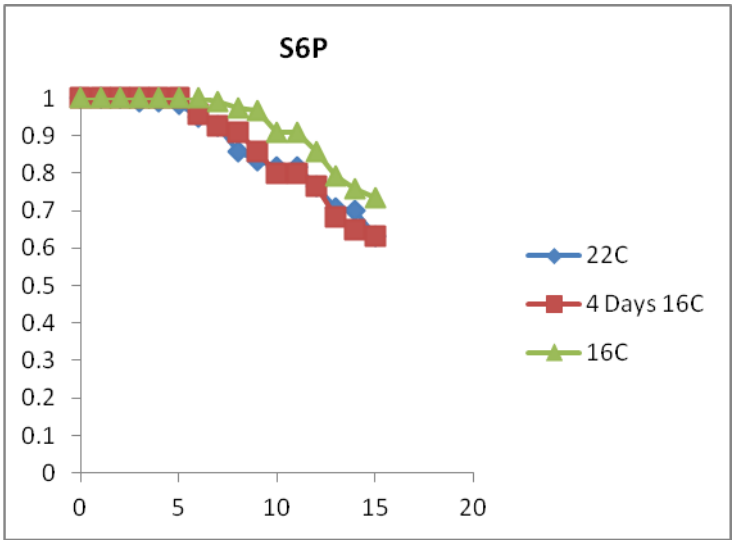

Figure 4. **(22C vs 16C: p = 0.0473, 22C vs 4d 16C: p= 0.9948, 4d 16C vs 16C: p = 0.0401)**This graph shows the longevity (in terms of days) of animals treated with double-stranded S6P RNA. Three treatments are shown: animals exposed continuously to 16°C, animals exposed continuously to 22°C, and animals exposed to 16°C for 4 days then exposed to 22°C for the remainder. The animals kept at 16°C for the first four days of their life, and then moved to an incubator kept at 22°C (denoted by the red line) show a closer survivorship to the animals kept at 22°C (blue line) unlike the untreated animals in figure 1.

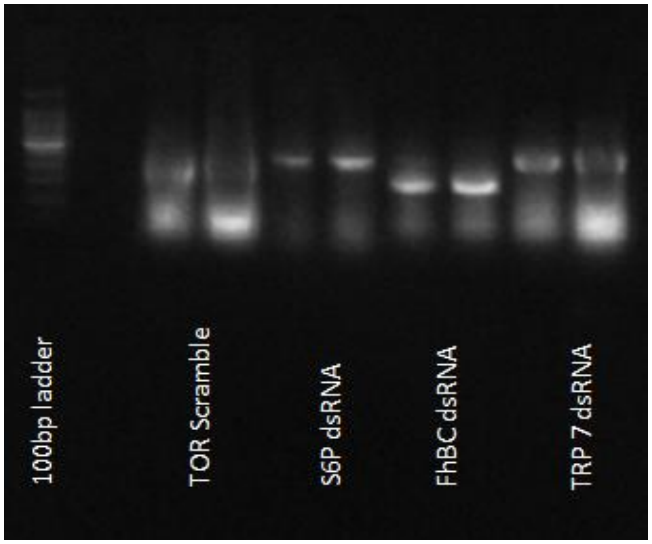

Figure 5. Gel electrophoresis: 20 minutes at 120V in 2% agarose gel. Bands show the approximate size of dsRNA products for each gel. The appearance of strong bands of S6P dsRNA and FhBC dsRNA is indicative of successful dsRNA production. Overexposure of TOR Scramble dsRNA and TRP7 dsRNA led to some discoloration, but the production of dsRNA in these cases was also verified. Bright lower bands are suspected to be the product of excess primer.

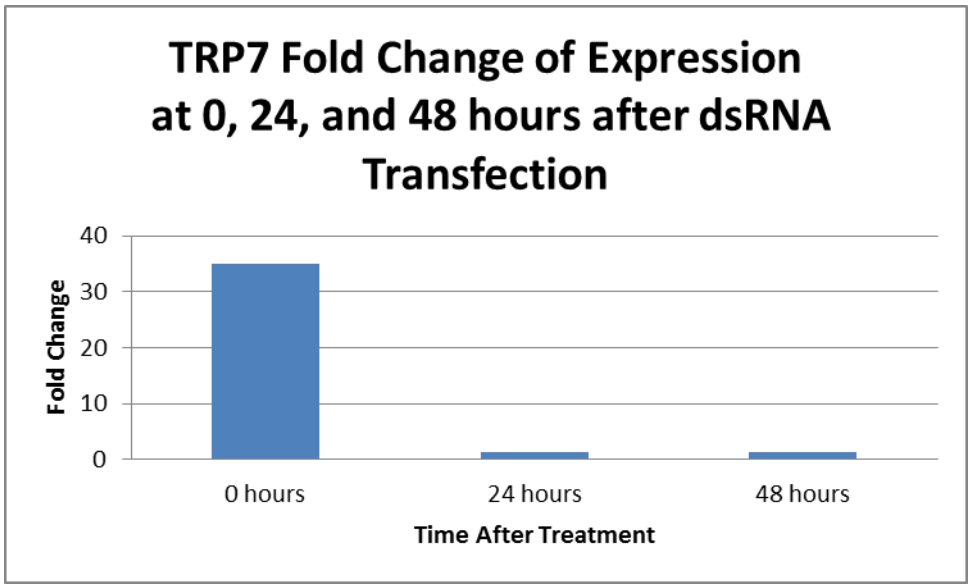

Figure 6. qPCR results were analyzed using the ΔΔCt method to find the fold change seen above. At 0 hours, there was a dramatic increase in fold change of TRP7 expression. At 24 hours, this fold change greatly decreased, but still does not demonstrate knock-down of the gene of interest. We can seen however, that the dsRNA has likely been digested by 24 hours after tranfection.

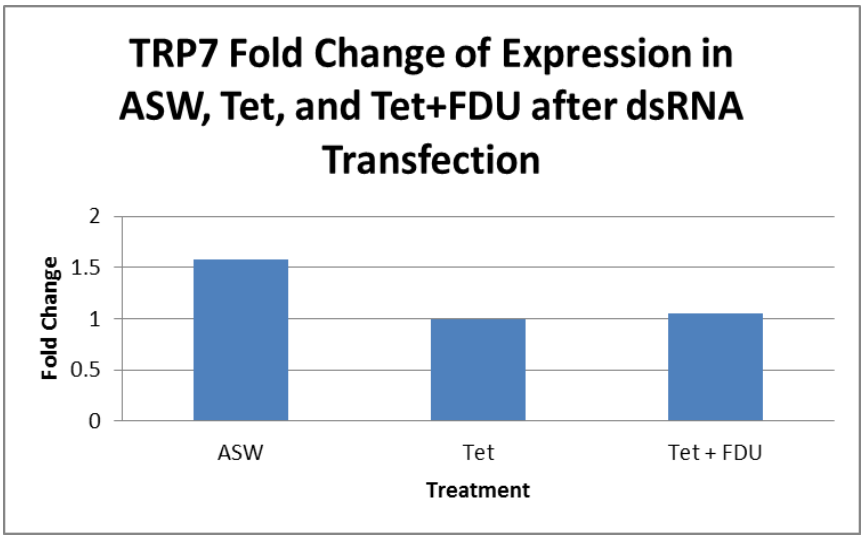

Figure 7. qPCR results were analyzed using the ΔΔCt method to find the fold change seen above. Animals were raised for 24 hours after transfection in artificial salt water, with food (*Tetraselmis suecica*), and with both food and FDU, the last treatment being typical in normal experimentation.

Gene	Abreviation	GB Accession	Forward	Reverse
Ribosomal Protein S6 Polypeptide 2	S6P	GARS01003002.1	GCGATGACGAC AATATCACG	TCCAACTGCATG AACAGCTC
Transient Receptor Potential Cation Subfamily Member 7	TRP7	GARS01012197.1	CAATGTTTTGG TCCTTATTTGG	CCAAATGGAATT GATCCGATA
Forkhead Box C-like Protein	FhBC	GARS01006072.1	AACGACTGCTT CTGCAAGGT	GACCGAAAACG ACGAGTAGG

Table 1. Three genes were explored in this experiment. The table above indicates the full gene name, GB Accession, and both sets of primers used to produce dsRNA.